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PQQ glucose dehydrogenase with novel electron transfer ability

Junko Okuda and Koji Sode*

Department of Biotechnology, Faculty of Technology, Tokyo University of Agriculture and Technology, 2-24-16 Nakamachi, Koganei, Tokyo 184-8588, Japan

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Abstract

PQQ glucose dehydrogenase from *Acinetobacter calcoaceticus* (GDH-B) is one of the most industrially attractive enzymes, as a sensor constituent for glucose sensing, because of its high catalytic activity and insensitivity to oxygen. We attempted to engineer GDH-B to enable electron transfer to the electrode in the absence of artificial electron mediator by mimicking the domain structure of the quinohemoprotein ethanol dehydrogenase (QH-EDH) from *Comamonas testosteroni*, which is composed of a PQQ-containing catalytic domain and a cytochrome *c* domain. We genetically fused the cytochrome *c* domain of QH-EDH to the C-terminal of GDH-B. The constructed fusion protein showed not only intra-molecular electron transfer, between PQQ and heme of the cytochrome *c* domain, but also electron transfer from heme to the electrode, thereby allowing the construction of a direct electron transfer-type glucose sensor.

Keywords: PQQ glucose dehydrogenase; Cytochrome; Electron transfer; Glucose sensor; Direct electron transfer type sensor

Several dehydrogenases harboring pyrroloquinoline quinone (PQQ) as their prosthetic group have been reported, such as glucose dehydrogenases, ethanol dehydrogenases, and methanol dehydrogenases[1–5]. They are divided into two categories, quinoprotein (PQQGDHs, PQQMDH, and type I PQQADH) and quinohemoprotein (types II and III PQQADH), the latter containing an additional prosthetic group, a heme *c* moiety, together with PQQ (the latter containing an additional heme *c* prosthetic group together with PQQ). These quino- and quinohemoproteins form protein complexes composed of catalytic subunits, with PQQ as their redox center, and electron acceptors, such as cytochrome *c*, which transfer electrons from reduced PQQ to the respiratory chain. The 3D structure of the quinohemoprotein ethanol dehydrogenase (QH-EDH) from *Comamonas testosteroni* was recently determined [6]. This enzyme has two domains separated with a peptide linker region, an eight-bladed β propeller fold catalytic domain containing PQQ, and a cytochrome *c* domain that is located at the C-terminal region. The heme is located on top of the catalytic site of the first domain, thereby allowing smooth electron transfer from the catalytic site via heme to the external electron acceptor.

Due to their superior electron transfer ability, such heme-containing, multicofactor dehydrogenases, consisting of a FAD- or PQQ-harboring catalytic subunit and a heme containing electron transfer subunit/domain, were recently shown to display direct electron transfer with the electrode [7–9].

The direct electron transfer has been investigated as the ultimate enzyme sensor format, allowing the direct monitoring of the catalytic reaction by the electrode. Unfortunately, only a limited number of enzymes, such as multicofactor enzymes, are capable of direct electron transfer to electrode.

Among the numerous dehydrogenases, the water soluble PQQGDH from *Acinetobacter calcoaceticus* (GDH-B) is one of the most industrially attractive enzymes. GDH-B has been expected to become a major sensor constituent for glucose sensing because of its high catalytic activity and insensitivity to oxygen. GDH-B is a basic (p*I* = 9.5), homodimeric enzyme. Each monomer (50 kDa, 454 residues) binds one PQQ molecule and three calcium ions and is folded into six four-stranded β sheets (β propeller fold) [10]. In the GDH-B structure,

* Corresponding author. Fax: +81-423-88-7027.
E-mail address: sode@cc.tuat.ac.jp (K. Sode).

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the PQQ prosthetic group is buried deeply within the protein shell. The direct electrochemical recycling of the prosthetic group by the electrode surface is therefore not observed.

In order to create a GDH-B capable of direct electron transfer to the electrode, we therefore attempted to construct a GDH-B–cytochrome *c* fusion protein. Considering the homologies between the scaffolds of the GDH-B catalytic subunit and the QH-EDH catalytic domain, we genetically fused the C-terminal cytochrome *c* domain of QH-EDH to the C-terminus of GDH-B. The resulting fusion protein was named QH-GDH. This paper describes the construction and characterization of QH-GDH and demonstrates its potential application for glucose sensor construction.

Materials and methods

Materials. Recombinant GDH-B and cytochrome b_{562} (cyt b_{562}) were prepared using *Escherichia coli* DH5α as host strain as previously described [11,12].

Construction of QH-GDH expression vector. The GDH-B structural gene (*gdh*B) without the stop codon and the electron transfer domain of QH-EDH were amplified by PCR from the genomes of *A. calcoaceticus* LMD79.41 and *C. testosteroni* ATCC15667, respectively. The following oligonucleotide primers used were designed to incorporate unique restriction enzyme recognition sites at their 5′ ends: GGCCA TGGATAAACATTTATTGGCTAAAATTGCTTTAT (*gdh*B, sense) [13], GGGGGAGCTCCTTAGCCTTATAGGTGAAC (*gdh*B, antisense), GGGGGAGCTCGGCAAGGCCAGGATGCCGGA (*ghedh* cyt *c* domain, sense), and antisense GGGGAAGCTTTCAGGGCT TGGGCCGGATGG (*qhedh* cyt *c* domain, antisense). PCR products were inserted into the multi-cloning site of the expression vector pTrc99A (Amersham Biosciences, Sweden) and the resulting QH-GDH expression vector was named pGBET.

Expression and purification. The constructed QH-GDH expression vector was co-transformed with pEC86 into *E. coli* strain DH5α (DH5α/pGBET, pEC86). pEC86, which was kindly donated by Professor Thöney-Meyer, ETH Switzerland [14], expresses the *E. coli ccmABCDEFGH* genes essential for the maturation of cytochrome *c* under the Km promoter. DH5α/pGBET, pEC86 was cultivated in L broth under semi-aerobic conditions at 30 °C for 10 h. The harvested cells were resuspended in 10 mM potassium phosphate buffer, pH 7.0, and disrupted by French press (110 MPa). The lysate was then ultracentrifuged (160,500*g*, 1.5 h, 4 °C) and dialyzed in 10 mM potassium phosphate buffer, pH 7.0. The resulting supernatant was applied to a Resource S cation exchange column (Amersham Biosciences) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The enzyme was eluted with a linear gradient of 5–150 mM NaCl in 10 mM potassium phosphate buffer, pH 7.0.

Enzyme assay. Enzyme assays were carried out as reported previously using 0.06 mM DCIP and 0.6 mM PMS [13,15,16]. One unit of GDH is defined as the amount of enzyme that oxidizes 1 μmol of glucose equivalent per minute under the standard assay conditions. Soluble protein was estimated by the method of Lowry et al. [17] using bovine serum albumin as the standard.

Electrochemical measurement. Electrochemical measurements were carried out as previously reported [18] with some modifications. QH-GDH electrodes were made by mixing 500 or 250 U QH-GDH in 20 mM Mops buffer, pH 7.0, with carbon paste (0.5 g graphite powder mixed with 0.3 ml paraffin liquid, BSA, West Lafayette, USA). GDH-B electrodes were made by mixing the carbon paste with 500 U GDH-B and an equimolar amount of cyt b_{562} or the same mass of BSA in 20 mM Mops buffer, pH 7.0. The mixtures were lyophilized and packed into the ends of carbon paste electrodes (3 mm in diameter, BAS) and stored in 20 mM Mops buffer, pH 7.0, at 4 °C until use. Each measurement was carried out in 20 mM Mops buffer, pH 7.0, containing 1 mM CaCl$_2$ for stabilization of the holo enzyme form. The applied potential for the calibration curve measurements was +300 mV vs. Ag/AgCl.

Results

Expression, purification, and characterization

We constructed the QH-GDH expression vector by fusing the structural genes of GDH-B and cytochrome *c* domain of QH-EDH via extra restriction enzyme site (*Sac*I) (Fig. 1). The 24-amino acid residue linker region between GDH-B and the cytochrome *c* domain was derived from the native structure of QH-EDH. Expression in *E. coli* of cytochrome *c* containing covalently bound heme requires the expression of *ccm* genes. Because the expression of *ccm* is strictly repressed under aerobic conditions, we co-transformed a compatible vector containing *ccm* genes under the tetracycline promoter for constitutive expression of *ccm*. The transformants harboring either of these vectors were yellowish whereas transformants harboring both the fusion enzyme-expressing and the *ccm*-expressing vectors were pink colored, suggesting the production of mature cytochrome *c*. Furthermore, transformed cells showed greater GDH activity than the host cells, indicating that the enzyme was expressed in the active form.

GDH-B and QH-EDH are highly basic enzymes, with calculated p*I* 8.85 and 8.99, respectively. In contrast, the cytochrome *c* domain is an acidic peptide, with a calculated p*I* of 5.65. The calculated p*I* value of QH-GDH is 8.6. QH-GDH elutes faster (0.1 M NaCl) than GDH-B (0.13 M NaCl) during cation exchange chromatography (data not shown). The surface of QH-GDH thus appears to be slightly more acidic than wild-type GDH-B, indicating that the acidic character of the cytochrome *c* domain affects the elution pattern. SDS–PAGE of

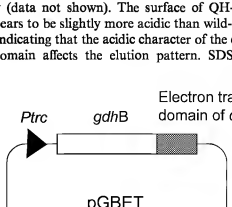

Fig. 1. Expression vector for QH-GDH.

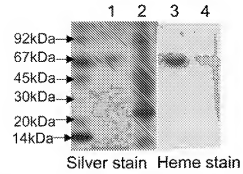

Fig. 2. SDS–PAGE of soluble fraction and eluted fractions of cation exchange chromatography. Lane 1, silver stained purified QH-GDH; lane 2, silver stained soluble fraction; lane 3, heme stained purified QH-GDH; and lane 4, heme stained soluble fraction.

purified QH-GDH (Fig. 2) shows a single band by both silver and heme staining. The MW of the fusion protein on SDS–PAGE (approximately 65 kDa) agrees well with the calculated MW of 62.4 kDa.

Analysis of the kinetic parameters of purified QH-GDH, summarized in Table 1, shows that its catalytic properties are almost identical to those of wild-type GDH-B. The K_m values of QH-GDH and GDH-B for glucose are 23 and 25 mM, respectively. The GDH activity of purified QH-GDH had a $k_{cat} = 3178\ s^{-1}$, which is similar to that of GDH-B [19]. The substrate specificities of the two enzymes were also similar.

The heme staining after SDS–PAGE indicated that this 65-kDa protein with GDH activity harbored a heme molecule. Addition of sodium hydrosulfite reduced the purified fusion protein, resulting in a UV/Vis spectrum typical for the reduced form of *c*-type cytochrome (Fig. 3A), showing peak wavelengths at 416, 522, and 551 nm. These results confirm that QH-GDH harbors heme and functions as cytochrome *c*.

In order to investigate the intra-molecular electron transfer between PQQ and the cytochrome *c* domain of QH-GDH, glucose was added to the oxidized enzyme form. The addition of glucose resulted in a spectrum change from the oxidized form of cytochrome *c* to the reduced form (Fig. 3B). These results suggest that QH-GDH shows GDH activity, contains a cytochrome *c* domain, and is also able to transfer electrons from the PQQ redox center to the cytochrome *c* domain.

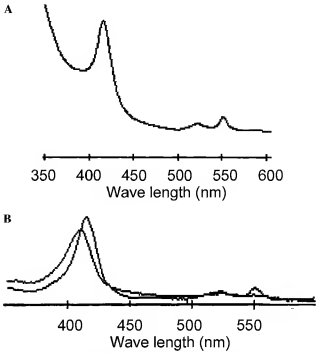

Fig. 3. Absorbance spectrum of QH-GDH (A) reduced by sodium hydrosulfite or (B) before and after addition of glucose.

Enzyme electrode

We then investigated the ability of QH-GDH to transfer electrons to the electrode. An electrode was constructed by mixing QH-GDH with carbon paste, and its current increase was investigated in the absence of an artificial electron acceptor. This electrode showed a rapid response to the addition of substrate and the sensor signal reached steady state current within 10 s after substrate addition (Fig. 4). The observed current increase was also found to be proportional to glucose concentration as well as to the amount of enzyme used (Fig. 5). The minimum detectable concentration of 0.01 mM for the enzyme sensor employing the QH-GDH fusion protein was derived from the calibration curve. The sensitivity of the sensor was $131.8\ \mu A\ M^{-1}\ cm^{-2}$.

In sharp contrast, no current increase was observed from electrodes immobilizing GDH-B and an equimolar

Table 1
kinetic parameters of QH-GDH and GDH-B

	QH-GDH			GDH-B[a]		
	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m
Glucose	23	3057	133	25	4610	184
Maltose	15	1721	114	26	2305	89
Lactose	19	1584	82	19	1982	105

[a] Kinetic parameters of GDH-B were extracted from Ref. [13].

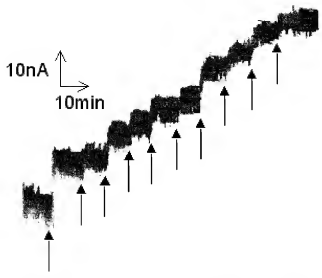

Fig. 4. Response curve of 750 U QH-GDH electrode. Each arrow indicates the introduction of substrate into reaction vessel.

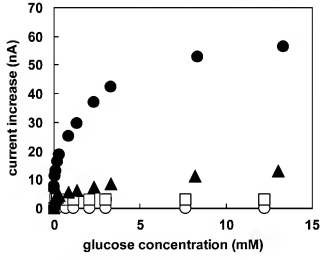

Fig. 5. Calibration curves of QH-GDH, GDH, and GDH co-immobilized with equimolar cytochrome in 20 mM Mops, pH 7, containing 1 mM $CaCl_2$ at 25 °C. Applied potential was +300 mV vs. Ag/AgCl. QH-GDH 750 U, closed circle; QH-GDH 375 U, closed triangle; GB/cyt b_{562}, open square; GB/BSA, open circle.

amount of cyt b_{562} or the same protein mass of BSA. We have previously reported that co-immobilization of GDH-B with cyt b_{562} showed a greater response to glucose than with cytochrome *c* from horse heart [20]. We had concluded that cyt b_{562} is more appropriate for GDH-B than cytochrome *c*. We therefore selected cyt b_{562} as the control protein in the present study. Cyt b_{562} ($E = +189$ mV vs. SCE at pH 7.0 [21], MW = 11,780) was used as the heme-containing protein that can be used as the electron acceptor of GDH-B [18], although no specific interaction exists between GDH-B and cyt b_{562}. The presence of an equimolar amount of cyt b_{562} did not produce a sensor response to glucose. In the case of the fusion protein, which contains the same amount of cytochrome *c* domain, a strong sensor signal was shown. BSA was used as a control to ensure that the results do not reflect a protein stabilization effect on GDH-B during the immobilization process. The GDH-B electrode with co-immobilized BSA did not show any response, indicating that the observed sensor signal was not due to the increase in the quantity of protein in the electrode and that GDH-B did not show any ability to transfer electrons to the electrode.

These results confirm the ability of QH-GDH to transfer electrons from the PQQ redox center to the electrode via the cytochrome *c* domain fused to its C-terminal region. These results also confirm the possibility of creating a direct transfer-type glucose enzyme sensor based on the fusion protein.

Discussion

In the present study we attempted to engineer GDH-B to enable it to transfer electrons directly to the electrode in the absence of artificial mediators by mimicking the two-domain structure of QH-EDH. By fusing the cytochrome *c* domain of QH-EDH to the C-terminus of GDH-B, a new enzyme was created that possessed a cytochrome *c* domain at its C-terminus while showing GDH activity almost indistinguishable from that of GDH-B. The fusion protein showed not only intramolecular electron transfer, between PQQ and heme of cytochrome *c* domain, but also showed electron transfer from heme to the electrode, thereby enabling to construct direct electron transfer type glucose sensor.

Oubrie et al. [6] reported the crystal structure of QH-EDH and predicted the electron transfer pathway from PQQ to the heme molecule. They reported that intramolecular electron transfer from PQQ to heme in QH-EDH occurs via disulfide ring that is conserved in PQQ-dependent alcohol dehydrogenases. These specific amino acid residues are also expected to be required to transfer electrons from PQQ to heme in our fusion protein. However, the conserved disulfide ring in PQQADHs is absent in GDH-B. Further structural information or site-directed mutagenesis studies may help clarify the electron transfer pathway.

As native GDH-B does not transfer electrons from its redox center to the electrode and does not have its own electron transfer subunit, we previously investigated the availability of a redox protein as electron acceptor for this enzyme. We reported the utilization of cyt b_{562} from *E. coli* as the redox protein [18]. The presence of a large excess quantity of cyt b_{562} was required for the electron transfer from PQQ to heme. By co-immobilizing GDH-B and more than 100-fold molar ratio of cyt b_{562} onto the electrode, an enzyme glucose sensor was constructed that did not employ artificial electron mediator. The GDH-B/

cyt b_{562} electrode was considered to be a useful approach to realize the construction of an enzyme sensor without hazardous electron mediators. However, as we demonstrated in the control experiment, the presence of an equimolar amount of cyt b_{562} to GDH-B did not show a significant electron transfer to the electrode. The preparation of a GDH-B/cyt b_{562} electrode is therefore much more costly than the enzyme sensor immobilizing GDH-B alone. Furthermore, the increased quantity of protein on the sensor chip consequently limits the maximum quantity of GDH-B on the electrode, thereby limiting the sensor properties. In contrast, the fusion protein can be prepared by a similar process as GDH-B, and this molecule alone has the ability of direct electron transfer. The fusion protein is therefore the best candidate to realize an artificial mediator-free glucose enzyme sensor.

Such an electron transfer property of the fusion protein may expand the use of GDH-B as the enzyme for Continuous Glucose Monitoring (CGM) systems. Currently developed CGM systems employ glucose oxidase (GOD) as the enzyme, and detect the signal with traditional electrochemical sensing of liberated hydrogen peroxide or consumed dissolved oxygen. It is evident that the oxygen-dependent signal of GOD is affected by changes in dissolved oxygen. Furthermore, such GOD-based sensors employ artificial electron mediators that are not suitable for in vivo use. Considering both the oxygen insensitivity of GDH-B and the direct electron transfer ability of fusion enzyme, future application of this fusion enzyme for CGM systems is expected.

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